

1 **Establishment of *in vitro* tissue cultures from *Echinacea angustifolia* D.C. adult plants**
2 **for the production of phytochemical compounds**

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24 **Abstract**

25 The establishment of *in vitro* cultures of *Echinacea angustifolia* was obtained directly from
26 section of flower stalks of adult plants. The vegetative propagation was obtained directly from
27 section of flower stalks placed on a mineral MS basal medium with B5 vitamins
28 supplemented with 0.5 mgL⁻¹ 6-benzylaminopurine (BA) while callus regenerative masses
29 were established from leaf sections cultured on the same basal medium supplemented with 3
30 mg L⁻¹ BA and 0.5 mg L⁻¹ indole-3-butyric acid (IBA). The secondary metabolite contents of
31 shoots proliferating on different culture media and callus masses were compared with *in vitro*
32 or *in vivo* seedlings. The quali-quantitative LC-DAD-ESI-MS analyses both on the *n*-hexanic
33 and methanolic extracts demonstrated that significant production of caffeic acid derivatives,
34 echinacoside and alkamides from different *in vitro* *E. angustifolia* tissues was possible.
35 Choosing the appropriate plant material from different *in vitro* cultures, the plant metabolite
36 pathway might be addressed towards the alkamides or the caffeic acid derivatives
37 productions.

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39 **Keywords:** alkamides; flavonoids; caffeic acid derivatives; *Echinacea angustifolia*; flower
40 stalk; *in vitro* shoots; LC-DAD-ESI-MS.

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45 **Introduction**

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47 The genus *Echinacea* belongs to the *Asteraceae* family and has nine species (McGregor,
48 1968). At present, only three species are used in phytotherapy: *E. angustifolia* D.C. (De
49 Candolle) var. *angustifolia* (syn. *Rudbeckia angustifolia* L.), *E. pallida* (Nutt.) Nutt. and *E.*
50 *purpurea* (L.) Moench.. *Echinacea* spp. are native of North America (McGregor, 1968) and
51 belonged to the rich Pharmacopoeia of the native Americans, who had used them for
52 hundreds of years for infections, inflammations and insect bites (Lloyd, 1921).

53 The chemistry of *Echinacea* species is well-known and caffeic acid derivatives, flavonoids,
54 polyacetylenes, alkamides, pyrrolizidine alkaloids, polysaccharides and glycoproteins were
55 isolated and characterized. (Bauer and Foster, 1991; Bauer and Wagner, 1991; Bauer and
56 Reminger, 1989; Bauer *et al.*, 1989; Bauer *et al.*, 1988a).

57 In the last few years, the increased demand of natural remedies in Europe has caused an
58 enhancing industrial request in the production of standardized plant material and extracts.

59 *Echinacea* is an Extraeuropean genus and the certified plant material for propagation of *E.*
60 *angustifolia* is not available yet (Li, 1998). Moreover, plants of the genus *Echinacea* are
61 characterized by their difficult germination caused mainly by seed dormancy (Baskin *et al.*,
62 1992; Macchia *et al.*, 2001; Feghahati and Reese, 1994; Sari *et al.*, 2001).

63 Progress in medicinal plant clonal propagation has been requested, especially for species such
64 as *Echinacea* with an agricultural production not sufficient for the growing pharmaceutical
65 industry demand. For this purpose, it is important to develop a reproducible protocol to
66 cloning *E. angustifolia*.

67 Till now only few reports showed attempts concerning the *in vitro* procedures for the
68 establishment of *E. angustifolia* clones from adult plants (Harbage, 2001; Lakshmanan *et al.*,
69 2001).

70 However there are no reports of regeneration using flower stalk sections as an explant source
71 for this species.

72 Difficulties to promote *E. angustifolia* tissue cultures from selected adult plants were mainly
73 caused by the rosette habit of this species. The short internodes and the vegetative apices
74 located near the ground, caused initial contaminations difficult to eradicate. In this work a
75 regeneration protocols from flower stalk explants was established. The use of this type of
76 explants allowed to reduce contamination problems and could be linked to previous analysis
77 of plant during their vegetative growth. The selection of important medicinal species is an
78 essential step to improve the agronomic and pharmaceutical features in particular when a high
79 phenotypic variability is present as in plant belonging to *Echinacea* family Regarding the
80 biosynthesis of the typical secondary metabolites, the presence of echinacoside, caffeic acid
81 derivatives, and polysaccharides were shown only in cell suspension cultures from seedling
82 tissues of *E. angustifolia* (Smith *et al.*, 2002), but most of the works about the production of
83 caffeic acid derivatives, alkamides and anthocyanins were carried on mainly on extracts of
84 other species of the *Echinacea* family cultivated *in vitro* (Schollhorn *et al.*, 1993; Sicha *et al.*,
85 1991 Luczkiewicz and Cisowski, 2001 Luczkiewicz *et al.* 2002). Moreover, no data on the
86 influence of the origin of the *in vitro* plantlets and their multiplication over time on secondary
87 metabolites are available in the literature.

88 In this work *E. angustifolia* *in vitro* cultures were established from adult plants.
89 Extraction and LC-DAD-ESI-MS protocols were performed in order to evaluate the
90 main secondary metabolites production in shoots collected in different phases of the *in*
91 *vitro* culture. Secondary metabolites content was compared with that of greenhouse
92 flowering plants and with *in vitro* germinated seedlings.

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93 Materials and methods

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95 Plant material

96 Open field *E. angustifolia* mother plants were furnished by the Department of Agronomy of
97 the University of Pisa. Plants were transferred to a greenhouse and conditioned (phase 0 of
98 micropropagation process according with Debergh and Maene (1981) by spraying them twice
99 with 0.10 g/l Benomyl fungicide (Du Pont Agricultural Products, Wilmington, Delaware, UK)
100 every 15 days the shoots were cut after an additional period of five days without treatments
101 (Mensuali-Sodi et al. 1997). Leaf explants and flower stalks were employed as starting
102 material for tissue culture.

103

104 *Echinacea angustifolia* tissue cultures from adult plants

105 Leaves and flower stalks removed from mother plants were subjected to a first washing in tap
106 water for 16 hours then, after reducing explant size, they were sterilized in a 15% of sodium
107 hypochlorite (8% Cl active) aqueous solution stirred for 15 min followed by three final rinses
108 in sterile distilled water. Under laminar flow cabinet the flower stalks were cut in slices 1-2
109 mm thick and the leaf explant portions were excised containing the central vein (0.25 cm²).
110 Each type of explants was placed in a climatic chamber 25 ± 1°C either in the darkness or at
111 16 hours of photoperiod with irradiance of 50 µmol sec⁻¹m⁻². The culture medium employed
112 was the basal medium, named CH, consisting of MS macro and micro elements, B5

113 Vitamines (Gamborg, 1968), 300 mg L⁻¹ reduced Gluthatione (GSH), 500 mg L⁻¹ 2-(N-
 114 Morpholino) ethanesulfonic acid (MES), 30 g L⁻¹ sucrose, 7 g L⁻¹ agar, pH 5.8. Two
 115 arrangements of growth regulators were used: 0.01 mg L⁻¹ 1-naphtaleneacetic acid (NAA)
 116 plus 1 mg L⁻¹ BA, and 0.5 mg L⁻¹ BA. Both media were added with 0.3% of *Plant*
 117 *Preservative Mixture*, Plant Cell Technology Inc., U.S.A. (PPM).
 118 *E. angustifolia* regenerated shoots were sequentially subcultured in vessels containing CH
 119 medium with 0.25 mg L⁻¹ (initial proliferating shoots: IP shoots) and 0.5 mg L⁻¹ BA (axillary
 120 proliferating shoots: AP shoots) interleaved by the CH medium with half mineral strength,
 121 vitamins and hormones free, 15 g L⁻¹ sucrose and 5 g L⁻¹ active charcoal.
 122 Leaves from *in vitro* shoots derived from flower stalk regeneration were excised and explants
 123 (0.5 cm²) were cut from the middle area of the lamina. Two different culture media were
 124 used named CHe and CHe* containing basal medium CH added with 3 mg L⁻¹ BA and 0.5
 125 IBA or 6 mg L⁻¹ BA 1 mg L⁻¹ IBA respectively.
 126 Regenerated shoot were subcultured on the same basal medium added with 0.5 mg L⁻¹ BA in
 127 Magenta vessels (LR).
 128 All media tested in these experiments were sterilized by autoclaving at 121°C at 1 atm. for 20
 129 min.
 130 *In vitro* cultures were maintained in a growth chamber at 22 ± 1°C with an irradiance of 80
 131 µmol sec⁻¹ m⁻² and photoperiod of 16 hours.

132

133 ***Echinacea angustifolia* greenhouse plants**

134 *Echinacea angustifolia* D.C. achenes were obtained from Gargini Sementi S.n.c. (Lucca,
 135 Italy). Achenes were sowed in Petri dishes and incubated at 25 ± 1°C with a 16 h photoperiod
 136 (cool white fluorescent light 70 µmol m⁻² s⁻¹). To overcome seed dormancy the inoculated
 137 achenes were previously subjected to stratification at 4°C in the dark for 11 days in the
 138 presence of 1 mM ethephon (2-chloroethylphosphonic acid) (Macchia *et al.* 2001). After

germination seedlings were transplanted in multi-pots containing pit-perlite soil (50:50 v:v) under greenhouse conditions. Leaf samples were collected at the beginning of the flowering period.

***Echinacea angustifolia* tissue cultures from seedlings.**

Seeds from the same source above cited, pre-treated to avoid seed dormancy with the same procedure above described, were sterilized by immersion in 70% ethanol for 30 s followed by soaking in 15% sodium hypochlorite solution (8% active chlorine) containing two drops of Tween 20[®] for 18 min and followed by three rinses in sterile water. Achenes were sowed on half strength inorganic basic nutrient MS (Murashige and Skoog, 1965), sucrose (15 g L⁻¹), agar (7 g L⁻¹), without vitamins or growth regulators. The medium was sterilized by autoclaving at 121°C at 1 atm. for 20 min. After the cold stratification period sowed seeds were transferred to a growth chamber and maintained at 25 ±1°C with a 16 h photoperiod (cool white fluorescent light 70 µmol m⁻² s⁻¹).

To induce shoot proliferation, *in vitro* germinated seeds were deprived of the root system and placed on the basal medium CH and, to promote shoot proliferation, 0.5 mg L⁻¹ BA was used. *In vitro* cultures were maintained in a growth chamber at 22 ± 1°C with an irradiance of 80 µmol sec⁻¹ m⁻² and photoperiod of 16 hours.

Plant Experiments and Statistical analysis

Explants used for shoot induction from adult plants were positioned in Petri ø 6 cm dishes (5 explant/dish, 5 dishes/treatment). During the proliferation and growing phase explants were subcultured into 175 ml glass culture vessels (5 explants/vessel; 10 vessels/treatment) and in G7 Magenta vessels (6 explants/vessel; 5 vessels/treatment). Shoot number per explant and length during the proliferation were expressed as mean ± standard error.

164 All the experiments were repeated twice and all data were recorded after three weeks (one
165 subcultures).

166 *In vitro* and *in vivo* germination data were recorded on the day 2, 4, and 6 after the pre-
167 treatments which were used to avoid seed dormancy. Germination ability was expressed as
168 germination percentages on the total seeds; mean germination time (MGT) was calculated as
169 reported to Ellis e Roberts (1980):

$$\frac{\sum(t_i \cdot n_i)}{\sum n_i}$$

171 where t_i represents the day number from the root emission and n_i the seed number germinated
172 in each time intervals ($n=50$; 5 seeds/Petri dishes). As regards shoot induction from aseptic
173 seedlings, each explant was placed into a disposable 30 ml vial ($n=25$). Germination
174 percentages, MGT, shoot number and length during the proliferation from *in vitro* seedling
175 were recorded and reported as mean values \pm Standard Error (SE).

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177 **Phytochemical investigation**

178 **Chemicals**

179 LC grade Water, Acetonitrile, Methanol and Formic acid (Backer) were used for LC-DAD-
180 MS Liquid Chromatography Diode Array Detector Electrospray Ionization Mass analysis.
181 Commercial compounds were used as some reference materials: caftaric acid (**1**) (10 mg,
182 ChromaDex, lot: 01-03028-301), chlorogenic acid (**2**) (10 mg, Extrasynthese, lot: 327-97-9),
183 echinacoside (**3**) (10 mg, ChromaDex, lot: 01-05020-101), cichoric acid (**4**) (10 mg,
184 ChromaDex, lot: 00-03640-300) and, caffeic acid (**5**) (10 mg, Sigma Aldrich, lot: 60018). The
185 flavonoids [quercetin (**7**), luteolin (**8**), apigenin (**9**), kaempferol (**10**), *p*-cumaric acid (**11**),
186 betulinic acid (**12**), apigenin 7-*O*- β -glucoside (**13**), isorhamnetin 3-rutinoside (**14**)] used as
187 standard compounds were part of a home-made database of natural compounds, isolated and
188 identified by NMR and MS experiments in our laboratory (HPLC purity grade 97-98%).

Alkamide (6) [dodeca-2E,4E,8Z,10E-tetraenoic acid isobuthylamide] was isolated and characterized during a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy) (Table 1).

Sample preparation and LC-DAD-ESI-MS analysis

The aerial parts (1.3 g) of the *E. angustifolia* plant material were freeze-dried and extracted by ultrasonic apparatus with *n*-hexane and methanol in turn (100 ml x 2h, 3 times).

Each fraction was analysed by LC-DAD-ESI-MS. LC system consisted of a Surveyor Thermofinnigan liquid chromatograph pump equipped with an analytical Lichrosorb RP-18 column (250 x 4.6 mm i.d., 5mm, Merck), a Thermofinnigan Photodiode Array Detector and an ion trap LCQ Advantage mass spectrometer. The analyses were carried out by a linear gradient using water with 0.1% HCOOH (solvent A), CH₃CN (solvent B) from 10:90 v/v (B-A) to 70:30 (15 min) (flow 0.7 ml/min, run time 40 min). The spectral data from the DAD detector were collected during the whole run in the range 210-700 nm and the peaks were detected at 254 (alkamides, flavonoids) and 330 nm (caffeolquinic derivatives, flavonoids) for all analysed samples.

LC-ESI-MS analyses [negative ion mode for caffeoilquinic derivatives (1-5) and flavonoids (7-14), positive mode for alkamide (6)], SRM Selected Reaction Monitoring, TIC Total Ion Current, m/z 100 to 800 amu) were performed in the same chromatographic conditions using the specific ESI values for caffeic acid and alkamides (sheath gas flow-rate 62 arbitrary units, auxiliary gas flow 9 arbitrary units, capillary voltage -16 V and capillary temperature 280°C). The qualitative results of LC-DAD-ESI-MS were showed in Table 1. The amounts of the compounds (1-6) were estimated by using a multilevel external standard procedure:

Caftaric acid (1)	$Y = 1.0347 + 2.5974 \times 10^{-5} X$	$r = 0.9977$
Chlorogenic acid (2)	$Y = 1.9601 + 1.6762 \times 10^{-5} X$	$r = 0.9985$
Echinacoside (3)	$Y = 0.1024 + 6.5431 \times 10^{-5} X$	$r = 0.9966$

215	Cichoric acid (4)	$Y = 2.1424 + 1.2889 \text{ E-}05 \text{ X}$	$r = 0.9988$
216	Caffeic acid (5)	$Y = 1.7444 + 8.4818 \text{ E-}06 \text{ X}$	$r = 0.9994$
217	Alkamide (6)	$Y = 1.668 + 1.2277 \text{ E-}05 \text{ X}$	$r = 0.9978$

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219 | Results

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221 *Echinacea angustifolia* tissue cultures from adult plants

222 The contamination of *E. angustifolia* explants was a serious problem that required a lot of
223 time consuming tentative to establish a protocol for the mother plant management to pull
224 down the micro flora of these plants. Several treatments with fungicides on the *in vivo* mother
225 plants and the presence of the biocide PPM in the culture medium gave 40% of non-
226 contaminated explants, which was sufficient to start the culture. Callus formation was induced
227 from leaf explants cultured on both tested media in the in light and darkness, but the callus
228 developed on the BA medium was necrotic as that on the NAA with BA medium in the light.
229 The flower stalk slices give rise to cell proliferation when cultured on 0.5 mg L^{-1} BA in the
230 light (Table 2 and Fig. 1). These green calli showed purple spots which generally developed
231 shoot primordial so they could be considered as markers of the regeneration process (Table
232 2). The shoots derived from these spots were subcultured on the same medium (Table 3)
233 maintaining, at the end of the second subculture, their morphogenetic capacity (Table 3)
234 with a sustained growth of regenerating callus. The leaf explants cultured on medium with
235 NAA combined with BA in dark condition, developed a conspicuous white friable callus
236 during the successive subculture but didn't show shoot regeneration at all (Table 3). During
237 this induction phase, hyperhydricity occurred in the new developed shoots. To reduce this
238 phenomenon, the shoots were subcultured in the same medium with half BA amount (0.25 mg
239 L^{-1}) (initial proliferating shoots: IP shoots) (Table 4), but the successive subcultures showed a
240 progressive culture deterioration which encountered a high hyperhydricity again (Figure 2A).

241 The successive culture on medium with active charcoal t lacking growth regulators
242 determined the callus reduction at the shoot basal end, a drastic decrease of the shoot number
243 and an increase in shoot length and quality (Table 4). When hyperhydricity was reduced,
244 *Echinacea* shoots were cultured again on the initial medium with 0.5 mg l⁻¹ BA. During this
245 multiplication phase the explants produced a low number of new axillary shoots (axillary
246 proliferating shoots: AP shoots) (Table 4 and Figure 2B). These shoots, not only showed
247 normal leaf features, no hyperhydric symptoms and less callus amount at the basal end but
248 also were able to regenerate, *de novo* shoots from the central vein of the intact leaves (Figure
249 3).

250 An analogous high morphogenetic potential was observed when leaf sections excised from
251 AP shoots were cultured.

252 From the results summarized in Table 5 we can notice that CHe medium including BA 3
253 mgL⁻¹ and IBA 0.5 mgL⁻¹ produced a high percentage of callus with purple spots which
254 could be considered “differentiation spots” as above described. Growth regulators in a double
255 concentration (CHe*) didn’t improve the callus formation and shoot differentiation.
256 Regenerated shoots from the callus cultures on CHe medium were subcultured (Figure 4) on
257 the same basal medium CH with 0.5 mgL⁻¹ BA (leaf regenerated shoots: LR shoots). The
258 results described in Table 5 demonstrated that shoot regeneration from *in vitro* growth leaves
259 could provide a good regeneration rate (1 leaf portion: 16 new shoots) useful to increase the
260 *E. angustifolia* shoot biomass.

261

262 ***Echinacea angustifolia* from seedlings**

263 To compare the phytochemical contents of *in vitro* cultures with those of tissues from *E.*
264 *angustifolia* propagated by seeds, *in vitro* and *in vivo* seedling cultivations were established.
265 For this purpose, germination was tested to provide the starting material for the greenhouse
266 and the *in vitro* cultivation. Pre-treatment with ethephon together with stratification of *E.*

267 *angustifolia* achenes was useful as demonstrated by the high percentage of germination (
268 69.7± 4.03)and a low mean germination time (MGT, 2.47±0-11).
269 New shoots (Proliferating Seedling shoots: PS shoots) was obtained culturing *E. angustifolia*
270 seedling explants on the CH basal culture medium with 0,5 mg/l BA (2,1 ± 0.3 number of
271 shoots per explants with an average length of 1.72 ± 0.24cm). ▼

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272 **Phytochemical analysis**

273 *Echinacea* plant material was investigated for the production of secondary metabolites as
274 listed below:

- 275 • Shoots from flower stalk of *E. angustifolia* plants and collected in the different
276 culture phases (IP shoots, AP shoots and LR shoots);
- 277 • Shoots from *in vitro* germinated seedlings named Proliferated Seedlings (PS shoots);
- 278 • Leaves from green-house plants collected at the beginning of the flowering period
279 named (GH plants).

280 The *n*-hexane and methanolic extracts were obtained by ultrasonic apparatus from fresh plant
281 material after freeze-drying. LC-DAD-ESI-MS analyses were performed in order to evaluate
282 the production of alkamides, flavonoids and caffeolquinic derivatives.

283 The phytochemical screening was carried out by the comparison of the retention time, UV
284 and MS spectra for each peak in the extract samples with those of the reference compounds
285 (**1-14**) (Table 1). A summary of the quantitative results (µg/g dry plant material) for the selected
286 constituents (**1-6**, Fig. 5) in the analysed samples is given in Table 7.

287 Alkamide (**6**) was detected as the main constituent in the *n*-hexane extracts of *in vitro* IP
288 shoots and LR shoots. Phenolic acids (**1, 2, 4, 5**) and echinacoside (**3**) were produced in much
289 lower amounts in comparison with the alkamide (**6**) in the same samples. An opposite result
290 was observed in the AP shoots and *in vivo* GH plants which produced caffeic acid derivatives
291 especially. The AP samples reduced the production of alkamide (**6**) by half in comparison
292 with the IP samples, but they were characterized by a much larger amount of chlorogenic acid

293 (2), echinacoside (3), and cichoric acid (4). The *in vitro* proliferating seedlings (PS shoots)
294 contained only cichoric acid (4).

295

296 **Discussion**

297

298 The aim of this work was to perform a standardized protocol for the massive multiplication of
299 *E. angustifolia* plants able to synthesize their typical secondary metabolites.

300 In this work it was settled up the active shoot organogenesis of flower stalks from adult
301 plants: the possibility to use this type of explant from a particular medicinal plant, previously
302 selected for their yield during the vegetative phase, could be very convenient for the growers.

303 This method could be applied without causing damage or completely destroying individual
304 plants as it occurred when the apical buds were excised from the rosettes. Plant propagation
305 from flower stalks was employed for *in vitro* regeneration of several species (Bajaj et al,
306 1983; Tan Nhut et al., 2001; Martin, 2005) but it is an unusual techniques for tissue culture of
307 *Compositae* plants. Direct and indirect regeneration appeared at the same time on flower stalk
308 explants exposed to light on media supplemented with 0.5 BA. This type of explant produced
309 a a satisfied shoot proliferation comparable with those observed on petioles and leaves of *E.*
310 *purpurea* (Choffe et al.,2000; Koroch et al. 2002). During these experiments this phenomenon
311 occurred together with a high shoot hyperhydricity and with the development of abundant
312 callus at the shoot basal end as also observed by Lakshmanan et al. (2001) on *in vitro* seedling
313 leaves of different *Echinacea* species.

314 Hyperhydricity was considered as a physiological response to simultaneous stress factors
315 determined by the *in vitro* culture conditions as high cytokinin treatments combined with the
316 high relative humidity in the closed flask atmosphere (Kevers et al., 2003). Therefore, in order
317 to obtain *E. angustifolia* plantlets more suitable to develop a continuous multiple shoot

318 production, the BA content was halved. The procedure gave a temporary improvement of the
 319 culture but the successive subcultures on the same medium showed again a progressive
 320 deterioration . A mid-step culture phase using active charcoal (Debergh and Maene, 1981)
 321 was necessary to restore shoot quality. These plantlets, cultured again on 0.5 mg L⁻¹ BA CH
 322 basal medium restore completely the shoot quality but maintained a poor multiplication
 323 capacity (1.7 shoots per explant). This multi-step protocol, improved the quality of the shoots,
 324 their elongation and it reduced the callus production at the basal end, giving suitable explants
 325 for the successive proliferation phases. The choice to increase the *in vitro* biomass and the
 326 previous observations concerning the direct organogenesis on shoot foliage, lead to perform
 327 leaf cultures on media with auxins and cytokinins . Auxins alone or in combination with
 328 cytokinins has been frequently associated with the induction and expression of regeneration in
 329 plant tissue culture (Steward et al., 1964). Zobayed and Saxena (2003), observed somatic
 330 embryogenesis from leaves of *E. purpurea* *in vitro* seedling when IBA (0.5 mgL⁻¹) was used
 331 together with BA (2.5 mg L⁻¹). In this work the combination of analogous IBA with BA
 332 concentrations in the CHe medium was useful to produce a conspicuous biomass of shoot
 333 regenerating callus confirming that the use of IBA might be effective in regeneration of
 334 recalcitrant species to the *in vitro* culture (Zobayed and Saxena, 2003). Summarizing, the *in*
 335 *vitro* propagation process of *E. angustifolia* from flower stalk consisted of three distinct
 336 phases: an adventitious regeneration phase from stalk sections, an axillary proliferation phase
 337 of the previous regenerated shoots and an adventitious regeneration phase from leaf pieces of
 338 the axillary shoots. The shoots sampled from these different differentiation process were
 339 subjected to the phytochemical analysis. Concerning to the analysis of the active constituents,
 340 LC-DAD-ESI-MS screening was carried out on the *in vitro* and *in vivo* plant material to
 341 evaluate the production of the typical antioxidant constituents (**1-14**) of *Echinacea* species
 342 (Table 1). The results showed that none of the well-known in *Echinacea* adult plants
 343 Flavonoids (**7-14**) (Bauer and Foster, 1991; Bauer and Wagner, 1991; Bauer and Reminger,

1989; Bauer *et al.*, 1989; Bauer *et al.*, 1988a) were present in the different plant material analysed in this work.

A significant difference between shoots from seedlings growing *in vitro* (PS shoots) and *in vivo* (GH) was showed. A lower secondary metabolite production was observed in PS (in vitro proliferating seedlings) shoots in comparison with the GH plants and it could be related to the *in vitro* growth conditions. In particular, between all the antioxidant constituents (**1-14**), the *in vitro* seedling accumulated only cichoric acid.

The *in vitro* shoots deriving from adult plants in the axillary proliferation phase (AP shoots) were characterized by a high amount of caffeic acid derivatives. They showed the production of caftaric, chlorogenic, cichoric acids, and echinacoside but no caffeic acid. AP shoots are plantlets well developed and showed phenotypic features similar to the greenhouse plants, for this reason, both cultures produced a significant amount of caffeolquinic derivatives. The yields of these secondary metabolites were similar or higher than those reported in leaves of *E. angustifolia* adult plants showed in Table 7. In particular, echinacoside (**3**), the main active constituent found only in wild or cultivated *E. angustifolia* roots (Bauer and Wagner, 1991; Bauer, 1998), was accumulated in a similar amount in the leaves of the AP shoots. In addition it could be remarked that cichoric acid was the main bioactive constituent in AP shoots and it showed much higher yields (3.05 %) in comparison with those reported in Table 7 from different organs of *E. angustifolia* adult plants (Bauer and Wagner, 1991; Bauer, 1998).

On the contrary, shoot at the end of the initial proliferating phase (IP shoots) accumulated a lower amount of phenolic metabolites in comparison with AP shoots and with the greenhouse plants but were able to produce a large quantity of alkamide (**6**). In addition, it was also evident that leaf regenerating shoots (LR) lost the ability to synthesize caffeic acid derivatives since they produced almost exclusively alkamide (**6**). It is well-known that alkamides are present in the roots, leaves, and stalks of wild or cultivated *E. angustifolia* plants (Bauer, *et al.* 1989). In this work the initial shoots from flower stalk and the regenerating shoots from leaf

explants (IP and LR) supplied an amount (0.023 and 0.036 % respectively) of alkamides (6) comparable to the typical content of wild *E. angustifolia* leaves and stalks showed in Table 7. This behaviour might be due to the IP and LR shoots physiological status in which a primary metabolism, directed towards the regeneration process, was dominant rather than a secondary one. Moreover, the higher propagation rates observed in IP and LR, might cause an hyperhydric status as observed by other Authors (Kevers et al. 2003; Hazarika, 2006) . Hyperhydricity, considered as a stress response, might lead IP and LR shoots towards several biochemical changes associated with a different pattern of metabolite accumulation opposite to AP shoots and GH plants.

The AP shoots drastically reduced the production of alkamide (6) in comparison with the IP and LR shoots. Furthermore, the leaves of *E. angustifolia* GH plants produced at least ten folds lower amounts of alkamide than the IP and LR shoots.

In conclusion, from our knowledge, this is the first report on significant production caffeic acid derivatives and alkamides from *in vitro* regenerated shoots of *E. angustifolia*. It was pointed out how the micropropagation of *E. angustifolia* plantlets from adult plants and the careful development of the proper multiplication procedures could allow us to get plant biomass able to produce active compounds at a rate comparable to that of the original plants. The different *in vitro* conditions, affect the plant metabolite pathway operating as a switch eliciting for the alkamide or the caffeic acid derivatives production. Therefore, the shoot regeneration protocols developed in the current study permit to choice the best culture phase to produce either caffeic acid derivatives or alkamides.

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 Home-made database of natural
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488 **Figure captions**

489

490 **Fig. 1** The *E. angustifolia* flower stalk slices give rise to cell proliferation and shoot
491 regeneration when cultured on CH basal medium with 0.5 mg L⁻¹ BA in the light.

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493 **Fig 2** Distinct type of *E. angustifolia* shoots during the multi-step propagation phases.

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494 **A** : Initial proliferating shoots (IP shoots); **B**: Axillary proliferating shoots (AP shoots);

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496 **Fig. 3** *E. angustifolia* shoot regeneration from the leaf central vein. Shoots were cultured on
497 CH basal medium with 0.5 mg L⁻¹ BA.

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499 **Fig. 4** Leaf regenerating shoots (LR shoots) on the CH basal medium with BA (3 mg L⁻¹) and
500 IBA (0.5 mg L⁻¹) named CHe.

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502 **Fig. 5** Chemical structures of the selected constituents (**1-6**) of *E. angustifolia* leaf analysed
503 samples.

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Table 1

Home-made database of natural compounds, alkamide (6), flavonoids and caffeic acid derivatives (1-14), isolated and identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).

N°	COMPOUND	Rt (min)	UV (λ nm)	MW	Base peak m/z	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)
1	caftaric acid	6.80	210, 240, 294, 329sh	312	[M-H] ⁻ 311.1	179.1 [M-H-132] ⁻	23	135.2 [M-H-132-CO ₂] ⁻	32		
2	chlorogenic acid	10.2	234sh, 244, 297sh, 328	354	[M-H] ⁻ 353.1	191.1 [M-H-162] ⁻	25				
3	echinacoside	15.1	220, 247sh, 292sh, 330	786	[M-H] ⁻ 785.3	623.0 [M-H-glc] ⁻	30	477.1 [M-H-glc-rha] ⁻	32		
4	cichoric acid	18.8	210, 244, 295, 330sh	474	[M-H] ⁻ 473.1	310.8 [M-H-162] ⁻	23	179.0 [M-H-162-132] ⁻	23	135.1 (44) [M-H-162-132-CO ₂] ⁻	32
5	caffeic acid	13.4	220, 247sh, 292sh, 330	180	[M-H] ⁻ 179.1	135.2 [M-H-CO ₂] ⁻	33				
6	dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	45.4	235, 260	247	[M+H] ⁺ 248.3	149.1 [M+H-99] ⁺	33	121.1 [M+H-99-C ₂ H ₄] ⁺	30	105.9 (15) [M+H-99-C ₂ H ₄ -CH ₃] ⁺	33
7	quercetin	29.6	255, 267, 301sh, 298sh, 370	302	[M-H] ⁻ 301.2	179.1 [M-H-122] ⁻	40	151.0 [M-H-122-CO] ⁻	38		
8	luteolin	30.2	253, 267, 242sh, 291sh, 349	286	[M-H] ⁻ 285.2	241.2 [M-H-CO ₂] ⁻	48	199.7 [M-H-CO ₂ -41] ⁻	43		
9	apigenin	33.3	267, 269sh, 336	270	[M-H] ⁻ 269.4						
10	kaempferol	14.9	253sh, 266, 294sh, 322sh, 367	286	[M-H] ⁻ 285.3						
11	p-coumaric acid	52.0	223, 286	164	[M-H] ⁻ 163.2	119.1 [M-H-CO ₂] ⁻	31				

12	betulinic acid	52.0	220, 307	456	[M-H+HC OOH] ⁻ 501.2		
13	apigenin 7 O β glucoside	32.7	286, 333	432	[M-H] ⁻ 431.1	269.3 [M-H-gluc] ⁻	35
14	isorhamnetin 3 O rutinoside	18.6	253, 267sh, 306sh, 326sh, 370	624	[M-H] ⁻ 623.1		

Table 2

Influence of the plant growth regulators (PGR) and the growth conditions on the regeneration tendency and callus initiation from leaves and flower stalks of *E. angustifolia* adult plants.

PGR	explant	regeneration tendency		callus induction		callus amount*		callus colour	
		dark	light	dark	light	dark	light	dark	light
BA	flower stalk	none	direct from purple spots	none	yes	–	+++	–	green with purple spots, compact
NAA+BA	flower stalk	none	none	none	none	–	–	–	–
BA	leaf	none	none	yes	yes	++	++	necrotic	necrotic
NAA +BA	leaf	direct etiolated	none	yes	yes	++	++	white, friable	necrotic

*Scale to quantify callus amount; += scarcely developed at the explant margins, ++ =

medium developed covering half of the explant, +++ = largely developed covering all the explant

Table 3

Shoot regeneration (number and length of new formed shoots) and callus formation (colour and texture) from flower stalk and leaf explants in different growth conditions.

Data are presented as means \pm SE.

Explants and growth conditions	Subcultures	N°shoots/exp	Length (cm)	Callus amount *	Colour, texture
Flower stalk- Light (0.5 mg L ⁻¹ BA)	I	2.67 \pm 0,33	0.67 \pm 0,20	+++	green, compact
	II	3.00 \pm 0.58	0.73 \pm 0.15	+++	green, compact
Leaf - Dark (0.01 mg L ⁻¹ NAA+ 1 mg L ⁻¹ BA)	I	1.67 \pm 0.33	0.50 \pm 0.01	++	white, friable
	II	0.00	/	++	white, friable

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Table 4

Shoot proliferation (shoot number and length of new formed shoots), and callus development at the basal end of *E. angustifolia* shoots in different phases of the propagation process. Data are presented as means \pm standard error. AP: Growth Phase shoots; IP: Proliferation Phase shoots.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
<u>IP shoots:</u> BA (0.25 mgL ⁻¹)	2,36 ± 0,40	1,31 ± 0,14	+ / ++	friable light green
Active charcoal (5 g/L)	1,10 ± 0,06	1,63 ± 0,25	+	compact green
<u>AP shoots:</u> 0.5 mg L ⁻¹ BA	1,77 ± 0,79	1,66 ± 0,39	+	compact green

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

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Table 5

Callus formation and shoot regeneration of *in vitro* *E. angustifolia* leaf portions on CH basal medium with different amounts of growth regulators (CHe and CHe*) and proliferation of shoots derived from Che medium (LR shoots). Shoot proliferation was performed on CH basal medium with 0.5 mg L⁻¹ BA.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
<u>CHe</u> (3 mg L ⁻¹ BA + 0.5 mg L ⁻¹ IBA)	3.98 ± 0.69	0.54 ± 0.10	+++	Friable white + purple spots
<u>CHe*</u> (6 mg L ⁻¹ BA + 1 mg L ⁻¹ IBA)	2.72 ± 1.57	0.42 ± 0.08	+++	Friable white
<u>LR shoots</u> (0.5 mg L ⁻¹ BA)	3.60 ± 0.54	2.65 ± 0.36	+	Friable at the shoot base

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

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Table 6

LC-DAD-ESI-MS analysis of the aerial part of *E. angustifolia* selected plant material.

IP: initial proliferating shoots; AP: axillary proliferating shoots; LR: Leaf Regenerated shoots; PS: Proliferating seedlings; GH plants: plants cultivated in greenhouse. Standard error (n = 3)

COMPOUNDS ($\mu\text{g/g}$ dried plant)	<i>In vitro</i> proliferation from flower stalk			seedlings	
	IP shoots	AP shoots	LR shoots	PS shoots	GH plants
caftaric acid (1)	16.54 ± 0.17	2551.5 ± 33.6	—	—	4283.7 ± 24.3
chlorogenic acid (2)	54.61 ± 2.11	11230.5 ± 145.5	—	—	1176.4 ± 6.5
echinacoside (3)	47.34 ± 0.98	5813.3 ± 53.7	—	—	5991.1 ± 46.1
cichoric acid (4)	46.13 ± 1.23	30530.8 ± 456.0	—	110.63 ± 1.32	1534.3 ± 10.6
caffeic acid (5)	4.11 ± 0.09	—	16.78 ± 0.16	—	116.4 ± 2.45
alkamide (6)	235.63 ± 9.86	107.35 ± 3.39	367.95 ± 10.75	—	26.83 ± 0.94

Table 7

Data from literature on the main active substance yields (%) in different organs of *Echinacea angustifolia* plants (4; 29).

Plant material	Echinacoside	Cichoric acid	Alkamides	Flavonoides	Glicoproteines /polisaccarides (µg/mg)	Essential oil (%/fresh plant material)
leaves		0.1	0.001-0.03	0.38		< 0.1
flowers	0.1-1.0	0.15	0.001-0.03			< 0.1
stalks		0.05	0.001-0.03			< 0.1
roots	0.3-1.3	traces	0.009-0.151		220.01	< 0.1

Figure



Figure



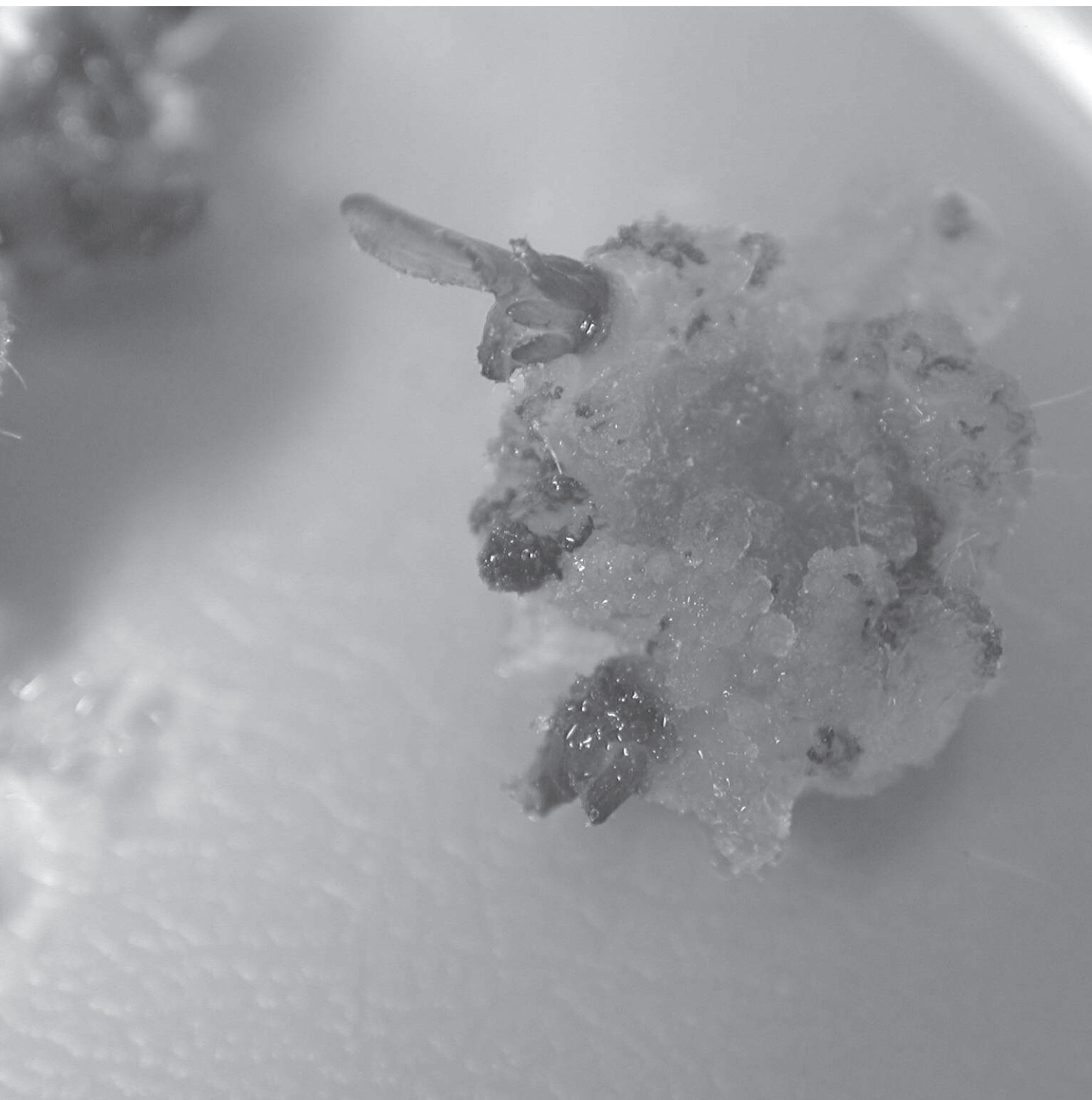
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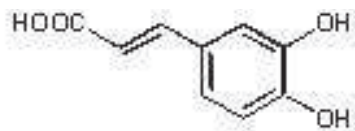
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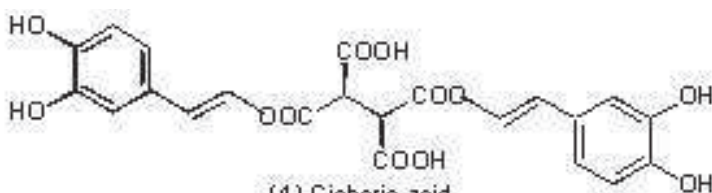
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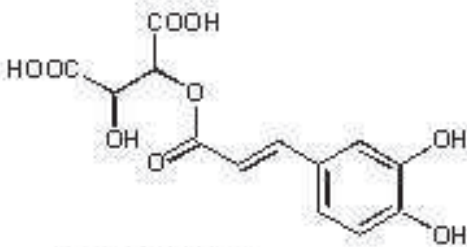
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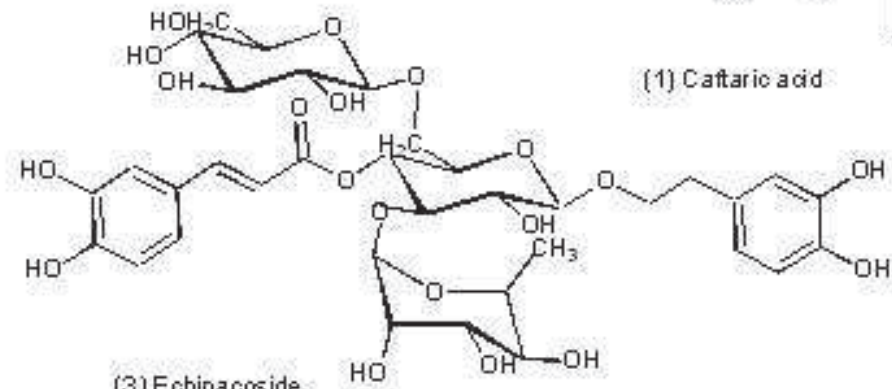
(5) Caffeic acid



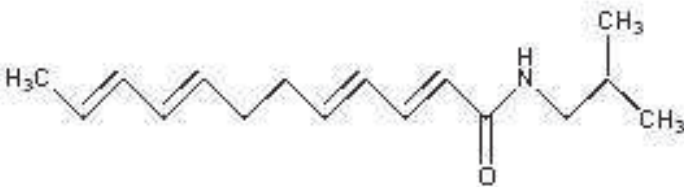
(4) Cichoric acid



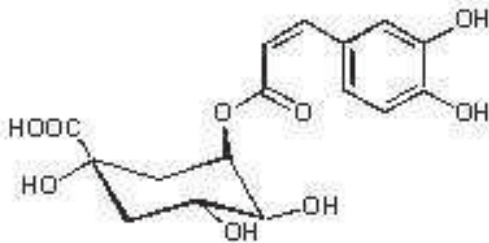
(1) Caftaric acid



(3) Echinacoside



(6) Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide



(2) Chlorogenic acid

Table 1

Home-made database of natural compounds, alkamide (6), flavonoids and caffeic acid derivatives (1-14), isolated and identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).

N°	COMPOUND	Rt (min)	UV (λ nm)	MW	Base peak m/z	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)
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2	chlorogenic acid	10.2	234sh, 244, 297sh, 328	354	[M-H] ⁻ 353.1	191.1 [M-H-162] ⁻	25				
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5	caffeic acid	13.4	220, 247sh, 292sh, 330	180	[M-H] ⁻ 179.1	135.2 [M-H-CO ₂] ⁻	33				
6	dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	45.4	235, 260	247	[M+H] ⁺ 248.3	149.1 [M+H-99] ⁺	33	121.1 [M+H-99-C ₂ H ₄] ⁺	30	105.9 (15) [M+H-99-C ₂ H ₄ -CH ₃] ⁺	33
7	quercetin	29.6	255, 267, 301sh, 298sh, 370	302	[M-H] ⁻ 301.2	179.1 [M-H-122] ⁻	40	151.0 [M-H-122-CO] ⁻	38		
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Table 2

Influence of the plant growth regulators (PGR) and the growth conditions on the regeneration tendency and callus initiation from leaves and flower stalks of *E. angustifolia* adult plants.

PGR	explant	regeneration tendency		callus induction		callus amount*		callus colour	
		dark	light	dark	light	dark	light	dark	light
BA	flower stalk	none	direct from purple spots	none	yes	–	+++	–	green with purple spots, compact
NAA+BA	flower stalk	none	none	none	none	–	–	–	–
BA	leaf	none	none	yes	yes	++	++	necrotic	necrotic
NAA +BA	leaf	direct etiolated	none	yes	yes	++	++	white, friable	necrotic

*Scale to quantify callus amount; += scarcely developed at the explant margins, ++ = medium

developed covering half of the explant, +++ = largely developed covering all the explant

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Shoot regeneration (number and length of new formed shoots) and callus formation (colour and texture) from flower stalk and leaf explants in different growth conditions. Data are presented as means \pm SE.

Explants and growth conditions	Subcultures	N°shoots/exp	Length (cm)	Callus amount *	Colour, texture
Flower stalk- Light (0.5 mg L ⁻¹ BA)	I	2.67 \pm 0,33	0.67 \pm 0,20	+++	green, compact
	II	3.00 \pm 0.58	0.73 \pm 0.15	+++	green, compact
Leaf - Dark (0.01 mg L ⁻¹ NAA+ 1 mg L ⁻¹ BA)	I	1.67 \pm 0.33	0.50 \pm 0.01	++	white, friable
	II	0.00	/	++	white, friable

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Table 4

Shoot proliferation (shoot number and length of new formed shoots), and callus development at the basal end of *E. angustifolia* shoots in different phases of the propagation process. Data are presented as means \pm standard error. AP: Growth Phase shoots; IP: Proliferation Phase shoots.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
<u>IP shoots:</u> BA (0.25 mgL ⁻¹)	2,36 \pm 0,40	1,31 \pm 0,14	+ / ++	friable light green
Active charcoal (5 g/L)	1,10 \pm 0,06	1,63 \pm 0,25	+	compact green
<u>AP shoots:</u> 0.5 mg L ⁻¹ BA	1,77 \pm 0,79	1,66 \pm 0,39	+	compact green

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Table 5

Callus formation and shoot regeneration of *in vitro* *E. angustifolia* leaf portions on CH basal medium with different amounts of growth regulators (CHe and CHe*) and proliferation of shoots derived from Che medium (LR shoots). Shoot proliferation was performed on CH basal medium with 0.5 mg L⁻¹ BA.

	N° shoot/exp	Length (cm)		Callus Amount*	Callus quality
<u>CHe</u> (3 mg L ⁻¹ BA + 0.5 mg L ⁻¹ IBA)	3.98 ± 0.69	0.54 ± 0.10		+++	Friable white + purple spots
<u>CHe*</u> (6 mg L ⁻¹ BA + 1 mg L ⁻¹ IBA)	2.72 ± 1.57	0.42 ± 0.08		+++	Friable white
<u>LR shoots</u> (0.5 mg L ⁻¹ BA)	3.60 ± 0.54	2.65±	0.36	+	Friable at the shoot base

*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Table 6

LC-DAD-ESI-MS analysis of the aerial part of *E. angustifolia* selected plant material.

IP: initial proliferating shoots; AP: axillary proliferating shoots; LR: Leaf Regenerated shoots; PS:

Proliferating seedlings; GH plants: plants cultivated in greenhouse. Standard error (n = 3)

COMPOUNDS ($\mu\text{g/g}$ dried plant)	<i>In vitro</i> proliferation from flower stalk			seedlings	
	IP shoots	AP shoots	LR shoots	PS shoots	GH plants
caftaric acid (1)	16.54 ± 0.17	2551.5 ± 33.6	—	—	4283.7 ± 24.3
chlorogenic acid (2)	54.61 ± 2.11	11230.5 ± 145.5	—	—	1176.4 ± 6.5
echinacoside (3)	47.34 ± 0.98	5813.3 ± 53.7	—	—	5991.1 ± 46.1
cichoric acid (4)	46.13 ± 1.23	30530.8 ± 456.0	—	110.63 ± 1.32	1534.3 ± 10.6
caffeic acid (5)	4.11 ± 0.09	—	16.78 ± 0.16	—	116.4 ± 2.45
alkamide (6)	235.63 ± 9.86	107.35 ± 3.39	367.95 ± 10.75	—	26.83 ± 0.94

Table 7

Data from literature on the main active substance yields (%) in different organs of *Echinacea angustifolia* plants (4; 29).

Plant material	Echinacoside	Cichoric acid	Alkamides	Flavonoides	Glicoproteines /polisaccarides (µg/mg)	Essential oil (%/fresh plant material)
leaves		0.1	0.001-0.03	0.38		< 0.1
flowers	0.1-1.0	0.15	0.001-0.03			< 0.1
stalks		0.05	0.001-0.03			< 0.1
roots	0.3-1.3	traces	0.009-0.151		220.01	< 0.1